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Fruit-specific lectins from banana and plantain

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Abstract. One of the predominant proteins in the pulp of ripe bananas (*Musa acuminata* L.) and plantains (*Musa* spp.) has been identified as a lectin. The banana and plantain agglutinins (called BanLec and PlanLec, respectively) were purified in reasonable quantities using a novel isolation procedure, which prevented adsorption of the lectins onto insoluble endogenous polysaccharides. Both BanLec and PlanLec are dimeric proteins composed of two identical subunits of 15 kDa. They readily agglutinate rabbit erythrocytes and exhibit specificity towards mannose. Molecular cloning revealed that BanLec has sequence similarity to previously described lectins of the family of jacalin-related lectins, and according to molecular modelling studies has the same overall fold and three-dimensional structure. The identification of BanLec and PlanLec demonstrates the occurrence of jacalin-related lectins in monocot species, suggesting that these lectins are more widespread among higher plants than is actually believed. The banana and plantain lectins are also the first documented examples of jacalin-related lectins, which are abundantly present in the pulp of mature fruits but are apparently absent from other tissues. However, after treatment of intact plants with methyl jasmonate, BanLec is also clearly induced in leaves. The banana lectin is a powerful murine T-cell mitogen. The relevance of the mitogenicity of the banana lectin is discussed in terms of both the physiological role of the lectin and the impact on food safety.

Key words: Fruit lectins – Jacalin – Lectin – Mannose – *Musa* – Plantain

Introduction

Recent advances in the biochemistry, molecular cloning and structural analysis of plant lectins allowed us to subdivide this heterogeneous group into seven families of structurally and evolutionarily related proteins (Van Damme et al. 1998). In the past, most of the research has been concentrated on the four classical families of plant lectins, namely the legume lectins, type-2 ribosome-inactivating proteins, chitin-binding lectins containing hevein domains and monocot mannose-binding lectins. There is a growing interest in the family of the so-called jacalin-related lectins and now there are firm indications that these lectins can play an important role in plant defense. Jacalin-related lectins were originally discovered in jack fruit (*Artocarpus integrifolia*) seeds, which contain large amounts of a Thomson-Friedenreich or T-antigen-specific agglutinin, called jacalin (Sastry et al. 1986). Later, similar lectins were identified in other *Artocarpus* species and in the osage orange (*Maclura pomifera*). All the *Artocarpus* lectins and the *Maclura pomifera* agglutinin are very similar with respect to their molecular structure ($\alpha 4\beta 4$), amino acid sequence and carbohydrate-binding specificity (Young et al. 1989, 1991). Due to their apparent confinement to a small taxonomic group, the jacalin-related lectins were considered to be a minor lectin family. However, this idea was abandoned after the identification of a mannose/maltose-specific lectin in rhizomes of the hedge bindweed (*Calystegia sepium*), which shares a high sequence similarity with the precursor of jacalin (Van Damme et al. 1996; Peumans et al. 1997). The discovery of the *Calystegia sepium* agglutinin (or Calsepa) in a typical representative of the family Convolvulaceae not only demonstrated the occurrence of a jacalin-related lectin outside the Moraceae but also revealed the existence of two subgroups within this lectin family. Jacalin and Calsepa have a different molecular structure ($[2\text{ kDa} + 13\text{ kDa}]_4$ versus $[16\text{ kDa}]_2$), and in addition, exhibit a totally different specificity [Galβ(1,3)GalNAc versus mannose]. Furthermore, jacalin and Calsepa strongly differ from each other regarding their biosyn-

The nucleotide sequence reported in this paper has been submitted to the Genbank/EMBL Data library under the accession number AF001527

Abbreviations: BanLec = banana lectin; Calsepa = *Calystegia sepium* agglutinin; HCA = hydrophobic cluster analysis; Heltuba = *Helianthus tuberosus* agglutinin; PlanLec = plantain lectin

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thesis and topogenesis. Jacalin is synthesized as a preproprotein, which undergoes a complex co- and post-translational processing involving the removal of a signal peptide, N-glycosylation, removal of an N-terminal propeptide and excision of a four-amino-acid residue linker between the mature α and β chains. Accordingly, one can reasonably assume that jacalin follows the secretory pathway and eventually accumulates in storage-protein vacuoles. In contrast, mature Calsepa corresponds to the entire open reading frame of the gene and hence undergoes no co- or post-translational processing. Due to the absence of a signal peptide it is assumed that Calsepa is synthesized and located in the cytoplasm. This hypothesis was recently confirmed by immunolocalisation studies of the lectin (data not shown). Calsepa was originally considered as a special member of the family of jacalin-related lectins. However, evidence is now accumulating that the cytoplasmic mannose-specific jacalin-related lectins are much more widespread than the vacuolar galactose-specific homologues, which are apparently confined to a few genera of the Moraceae family. A lectin similar to Calsepa has been found, indeed, in tubers of the Jerusalem artichoke (*Helianthus tuberosus*, Asteraceae; Van Damme et al. 1999). Recently, we have shown that a mannose-binding jacalin-related lectin is also induced in salt-stressed rice (*Oryza sativa*) plants (Zhang et al. 2000). In addition, evidence has been presented that the polypeptides of the so-called myrosinase-binding proteins from *Brassica napus* seeds and seedlings contain domains with a high sequence identity to the jacalin-related lectins (Geshi and Brandt 1998). Differential screening of cDNA libraries representing banana (*Musa acuminata*) pulp at different ripening stages also yielded a cDNA clone with sequence similarity to jacalin (Clendennen and May 1997). To check whether the putative jacalin-related lectin from banana (BanLec) corresponds to a previously described mannose-binding banana agglutinin (Koshte et al. 1990) the pulp lectin was re-investigated and the corresponding cDNA completely sequenced and analyzed.

The identification of BanLec as a novel member of the family of jacalin-related lectins is not only important because it would provide definitive proof that these lectins occur both in monocots and dicots but also because it would put the molecular evolution of this protein family in a new perspective.

Materials and methods

Plant material. Bananas (*Musa acuminata* 'Cavendish' cv. Grand Nain) and False Horn plantains (*Musa* ssp.) were purchased from a local store and kept at 20–25 °C until the pulp was completely softened (i.e. beyond peel color index 7).

Purification of the banana and plantain lectins. Peeled overripe bananas (5 kg) were immersed in a solution of 50 mM acetic acid, soaked overnight at 2 °C and homogenized with a mixer in a total volume of 20 l of the same solution. The homogenate was kept at 2 °C for 24 h to allow the foam to separate from the liquid. After removal of the foam the extract was poured through cheesecloth, adjusted to pH 3.0 with 1 N acetic acid and centrifuged at 9000*g* for 15 min. The supernatant was filtered through filter paper (Whatmann 3MM) and loaded onto a column of S Fast Flow (5 cm diameter, 10 cm long; 100 ml bed volume; Pharmacia) equilibrated with 20 mM acetic acid. Afterwards, the column was washed with 1 l of formate buffer (20 mM Na-formate, pH 3.8) and the bound proteins eluted in a single step with 200 ml of 1 M NaCl in formate buffer.

The lectin was isolated from the protein fraction desorbed from the S Fast Flow column by affinity chromatography on immobilized mannose. After loading the protein mixture onto a column of mannose-Sepharose 4B (2.6 cm diameter, 10 cm long; 50 ml bed volume) equilibrated with formate buffer containing 0.2 M NaCl, the column was washed with the same buffer until the A_{280} fell below 0.01, and the bound proteins eluted with 100 ml of 20 mM acetic acid. Then the pH of the lectin solution was adjusted to 3.8 and solid NaCl was added to a final concentration of 0.2 M. After standing overnight in the cold, the lectin solution was cleared by centrifugation (9000*g* for 15 min) and the affinity-chromatography step repeated. At the end of the second affinity chromatography the lectin was eluted with 0.1 M mannose in formate buffer, dialyzed against appropriate buffers and frozen at −20 °C until use. The total yield of affinity-purified lectin was about 50 mg.

The same procedure was followed to isolate the lectin from the pulp of ripe plantains. Starting from 4 kg of peeled plantains about 200 mg affinity-purified lectin was obtained.

Hemagglutination tests. Agglutination assays were carried out in small glass tubes in a final volume of 50 µl containing 40 µl of a 1% suspension of red blood cells and 10 µl of lectin solutions. To determine the agglutination titer, the lectin was serially diluted with 2-fold increments. Agglutination was assessed visually after 1 h at room temperature. Human and rabbit blood cells were treated with trypsin, as described previously (Peumans et al. 1997).

The carbohydrate-binding specificity of the lectin was determined by inhibition of agglutination of trypsinized rabbit erythrocytes. To 10-µl aliquots of serially diluted stock solutions of the inhibitors (glycoproteins, mono- and oligosaccharides, and methyl monoglycosides) 10 µl of a lectin solution (10 µg/ml) was added. After preincubation for 1 h at 25 °C, 30 µl of a 1% suspension of trypsinized rabbit erythrocytes was added and the agglutination evaluated after 1 h. The sugars tested were: glucose, galactose, galactosamine, N-acetylglucosamine, N-acetylgalactosamine, mannose, lactose, melibiose, fucose, arabinose, ribose, fructose, trehalose, sorbose, xylose, sucrose, maltose and sorbitol. All sugars tested were of the D-configuration except for L-fucose and L-sorbose. Glycoproteins tested were thyroglobulin, ovomucoid, fetuin, asialofetuin, mucin and asialomucin.

Analytical methods. Purified lectins were analyzed by SDS-PAGE using 12.5–25% (w/v) acrylamide gradient gels as described by Laemmli (1970). For N-terminal amino acid sequencing, purified lectins were separated by SDS-PAGE and electroblotted on a polyvinylidenedifluoride membrane. Polypeptides were excised from the blots and sequenced on a protein sequencer (model 477A; Applied Biosystems) interfaced with an on-line analyzer (model 120A; Applied Biosystems).

Total neutral sugar was determined by the phenol/H_2SO_4 method (Dubois et al. 1956), with D-glucose as standard.

Analytical gel filtration was performed on a Pharmacia Superose 12 column using phosphate-buffered saline containing 0.1 M galactose and 0.1 M mannose (to avoid possible binding of the lectins to the column) as running buffer. Due to the aberrant elution behavior of lectins, the molecular mass of native lectins was estimated using well-characterized members of the same lectin family, namely, *Helianthus tuberosus* agglutinin (Heltuba) (60 kDa) and Calsepa (32 kDa) as markers.

For the mannose-Sepharose column, mannose was coupled to Sepharose 4B by activation with divinylsulphone (1 ml/10 ml gel) in 0.5 M sodium carbonate pH 11, for 3 h at 25 °C. After activation, the gel was washed extensively with water. The coupling to mannose (100 mg/ml) was for 15 h at 37 °C in 0.5 M sodium

carbonate, pH 10. After coupling, the gel was washed thoroughly with water and the remaining activated groups blocked by incubation in 0.2 M Tris/HCl, pH 8.5, for 3 h at 25 °C.

Stability tests. Aliquots of purified lectins (10 μg/ml in 20 mM Tris-HCl (pH 7.5) containing 0.2 M NaCl) were heated in a waterbath for 5 min at different temperatures, quenched cold in ice and assayed for agglutination activity. Aliquots of the same lectin solutions were adjusted to different pH values (by adding 0.1 N HCl or 0.1 N NaOH). After 1 h at room temperature, samples were adjusted to 0.1 M Tris-HCl (pH 7.5), and assayed for agglutination activity. To determine resistance to proteases the lectin solutions were supplemented with 10 μg/ml trypsin or proteinase K. After incubation for 1 h at 37 °C, samples were assayed for agglutination activity.

Mitogenicity tests. Cell proliferation assays were carried out as previously described (Bemer and Truffa-Bachi 1996). Briefly, 2×10^5 spleen cells from 6- to 8-week-old C57BL/6 or BALB/c female mice (IFFA-CREDO, L'Arbresle, France) were cultured in 100 μl of RPMI 1640 medium (Life Technologies Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μg/ml streptomycin, 50 U/ml penicillin and 5×10^{-5} M 2-mercaptoethanol at 37 °C in a humidified air atmosphere of 5% CO_2. For the experiments, 96-well flat-bottom tissue-culture cluster plates (Costar) were used. Various amounts of BanLec were added at the onset of the culture. Concanavalin A was purchased from Sigma and was used at 2 μg/ml. T-cell proliferation was determined by the uptake of 9.25×10^6 Bq/well of [^{3}H]-thymidine (Amersham Pharmacia Biotech) added to the cultures 4 h before the end of the assay. Radioactivity was determined in a β-Plate liquid scintillation β-counter.

Sequencing of pBAN 3-32. The cDNA clone pBAN 3-32 resulted from a differential screening of cDNA libraries representing banana pulp at different ripening stages (Clendennen and May 1997). The sequence of pBAN 3-32 was completed by the dideoxy method (Sanger et al. 1977). The DNA sequences were analyzed using programs from PC Gene (Intelligenetics, Mountain View, Calif., USA) and Genepro (Riverside Scientific, Seattle, Wash., USA).

Southern blot analysis. Genomic DNA purification and Southern blot analysis were performed as detailed in May et al. (1995).

Methyl jasmonate treatment. Young banana (*Musa acuminata* L. cv. Giant Cavendish) plants (each containing four expanded leaves) were treated with methyl jasmonate for 3 d. To this end, young plants were transferred to a closed container (25 l) and 20 μl of a 1% (v/v) solution of methyl jasmonate in ethanol was added daily on a strip of filter paper. Controls were without methyl jasmonate. Samples of root and leaf material were collected and analyzed for the presence of lectin using agglutination tests and northern blot analysis of total RNA isolated from the plant tissue as described previously (Eggermont et al. 1996).

Molecular modelling. The amino acid sequence alignments were carried on a MicroVAX 3100 (Digital, Evry, France) using the ialign program of PIR/NBRF (Washington, D.C., USA). The program SeqVu (Gardner J 1995, The Garvan Institute of Medical Research, Sydney, Australia) was used to compare the amino acid sequences of the banana lectin BanLec with those of jacalin and other related lectins. MacClade (Maddison and Maddison 1992) was run on a Macintosh 5400/180 to build a parsimony phylogenetic tree relating the banana lectin to other lectins. Multiple amino acid sequence alignments based on Clustal W (Thompson et al. 1994) were carried out using SeqPup (Gilbert DG, Biology Dept., Indiana University, Bloomington, Ind., USA) and modified manually to build the phylogenetic tree.

The hydrophobic cluster analysis (HCA; Gaboriaud et al. 1987; Lemesle-Varloot et al. 1990) was performed to delineate the structurally conserved regions along the amino acid sequences of BanLec and Heltuba used as a model. The HCA plots were generated using the programme HCA-Plot2 (Doriane, Paris, France).

Molecular modelling of BanLec was performed on a Silicon Graphics O2 R10000 workstation, using the programmes InsightII, Homology and Discover (MSI, San Diego Calif., USA). The atomic coordinates of Heltuba (Bourne et al. 1999) were used to build the three-dimensional model of BanLec. Energy minimization and relaxation of the loop regions were carried out by several cycles of steepest descent and conjugate gradient using the cvff forcefield of Discover. The program TurboFrodo (Bio-Graphics, Marseille, France) was run on the workstation to draw the Ramachandran plots and perform the superimposition of the models. Cartoons were rendered using Molscript (Kraulis 1991).

Results

Detection and purification of lectins from the pulp of ripe bananas and plantains. Crude extracts from pulp of bananas and plantains (prepared by homogenization of 1 g tissue in 10 ml of 0.2 M NaCl and filtering the homogenate through glass wool) readily agglutinated trypsin-treated rabbit erythrocytes, the agglutination titers being 10 and 50, respectively. However, virtually all agglutinating activity was removed from the extract after centrifugation at 13 000*g* for 4 min, indicating that the lectin was present in an insoluble or complexed (with endogenous glycans) form. Due to the apparent insolubility under normal physiological conditions, a novel method had to be developed to achieve a high-yield purification of the lectins. Therefore, advantage was taken of the fact that the lectins are reversibly inactivated at low pH. When the pH of extracts was kept below 3.0, the lectins did not precipitate upon centrifugation. After concentration of the proteins on a cation-exchange column, the lectins remained in solution and could be purified by affinity chromatography on immobilized mannose. Following the procedure described in *Materials and methods*, the overall yield of affinity-purified lectin was 10 and 50 mg/kg fruit pulp for banana and plantain, respectively.

The purification procedure followed in this paper is simple in comparison to a previously described method which involved the addition of 0.1 M α-methyl-D-mannopyranoside to the extract, followed by defatting with Freon and dialysis for 100 h prior to affinity chromatography on Sephadex G-75 (Koshte et al. 1990). By virtue of its simplicity our novel method can easily be scaled up thus allowing the isolation of large amounts of the banana and plantain lectins.

Purification and partial characterization of the banana and plantain fruit lectins. The banana and plantain lectins (further referred to as BanLec and PlanLec, respectively) were identified on the basis of their agglutination activity and analyzed by gel filtration and SDS-PAGE to determine their molecular structure. The SDS-PAGE of both the reduced and unreduced lectins yielded a single polypeptide band of about 15 kDa (Fig. 1). Both native lectins eluted with an apparent M_r of about 30 kDa upon gel filtration on a

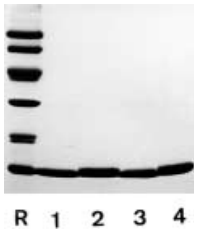

Fig. 1. Analysis of purified banana and plantain lectins by SDS-PAGE. Unreduced BanLec and PlanLec were run in *lanes 1* and *2*, respectively. Reduced (with β-mercaptoethanol) BanLec and PlanLec were loaded in *lanes 3* and *4*, respectively. Molecular mass reference proteins (*lane R*) were lysozyme (14 kDa), soybean trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and phosphorylase b (94 kDa)

Superose 12 column (in the presence of 0.1 M mannose to prevent interactions between the lectin and the matrix) using standard reference proteins as molecular weight markers. Since lectins often exhibit an unusual elution behavior upon gel filtration, the elution position of BanLec and PlanLec was compared to that of two other well-characterized lectins from the same protein family. These lectins were the dimeric Calsepa (2 × 16 kDa) and the tetrameric Heltuba (4 × 15 kDa). Since BanLec and PlanLec eluted at the same position as Calsepa (data not shown) we assume that both native lectins are dimers composed of two identical subunits of 15 kDa. More-accurate estimates by mass spectrometry yielded M_r values (±SD) of 14 426 Da ± 0.84 and 14 576 Da ± 4.63 for BanLec and PlanLec, respectively.

No covalently bound carbohydrate could be detected either in BanLec or in PlanLec. N-terminal amino-acid sequencing of BanLec yielded the single sequence NGAIK VGAWG GNGGS AFDMG.

Agglutination activity and carbohydrate-binding properties. Both BanLec and PlanLec readily agglutinated trypsin-treated rabbit erythrocytes, the minimal concentration required for agglutination being 0.25 μg/ml. In the same test, Calsepa and jacalin yielded values of 0.18 and 0.046 μg/ml, respectively. Trypsin-treated human erythrocytes irrespective of the blood group did not agglutinate in the presence of 100 μg/ml lectin.

The carbohydrate-binding specificity of BanLec and PlanLec was determined in some detail by hapten inhibition assays of the agglutination of rabbit erythrocytes. As shown in Table 1, the best inhibitor for BanLec was methyl α-mannopyranoside, followed by mannose and methyl α-glucoside, which were two and three times less effective, respectively. Trehalose, 2-deoxyglucose, fructose and maltose were 75% less effective inhibitors than methyl α-mannopyranoside. Glucose was only 25% as active as mannose. Of the animal glycoproteins tested, only mucin and asialomucin were potent inhibitors of BanLec and PlanLec (Table 1). Both glycoproteins yielded 50% inhibition at a concentration of 0.2 μg/ml.

Stability of BanLec and PlanLec. The stability of purified BanLec and PlanLec was investigated under different conditions of pH and temperature. Both lectins were stable over the pH range 2.5–12 and were heat-stable up to 70 °C. Treatment of BanLec and PlanLec with trypsin or proteinase K did not reduce the agglutinating activity of the lectins.

Table 1. Carbohydrate binding specificity of the banana and plantain lectin

Sugar/glycoprotein	Concentration required for 50% inhibition of:	
	BanLec	PlanLec
Thyroglobulin	8 μg/ml	8 μg/ml
Fetuin	250 μg/ml	250 μg/ml
Asialofetuin	250 μg/ml	250 μg/ml
Ovomucoid	>2000 μg/ml	>2000 μg/ml
Mucin	0.2 μg/ml	0.2 μg/ml
Asialomucin	0.2 μg/ml	0.2 μg/ml
Mannose	20 mM	20 mM
Trehalose	40 mM	40 mM
2-Deoxyglucose	40 mM	40 mM
Maltose	40 mM	40 mM
Fructose	40 mM	40 mM
Sucrose	>100 mM	>100 mM
Glucose	80 mM	80 mM
Methyl-α-mannopyranoside	10 mM	10 mM
Methyl-α-glucopyranoside	30 mM	30 mM
Methyl-α-galactopyranoside	>100 mM	>100 mM

Splenic lymphocyte response to BanLec. Preliminary experiments were performed on C57BL/6 and BALB/c spleen cells using different doses of BanLec ranging from 0.25 to 8 μg/ml. Four-hour thymidine pulses were performed daily. The concentration of BanLec giving the optimal thymidine uptake was 1 μg/ml and the peak of the response was observed at day 3 for the two strains of mice (data not shown). However, as shown in Fig. 2, the level of the response was different in the two mice strains. Thymidine uptake in C57BL/6 spleen cells was 2- to 3-fold lower than in BALB/c spleen cells. In

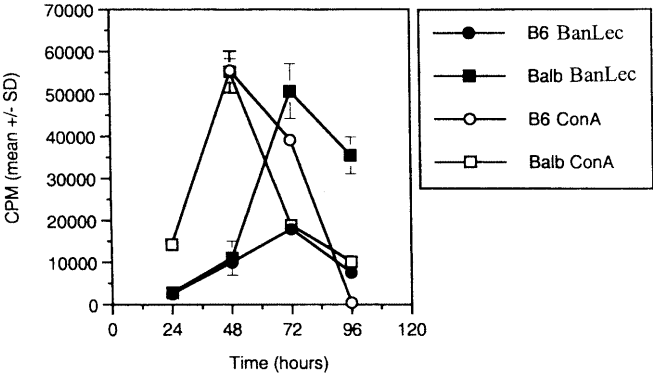

Fig. 2. Response of splenic lymphocytes to BanLec. Approximately 2×10^5 C57BL/6 (B6) or BALB/c spleen cells were stimulated by 1 μg/ml of BanLec or 2 μg/ml of ConA for 96 h in complete RPMI medium. Cells were pulsed with 0.94×10^4 Bq of [^{3}H]thymidine for the final 4 h. The background uptake of the unstimulated cell culture was below 200 cpm. Results are presented as the mean of triplicate determinations (±SD). The data are representative of one out of two independent experiments

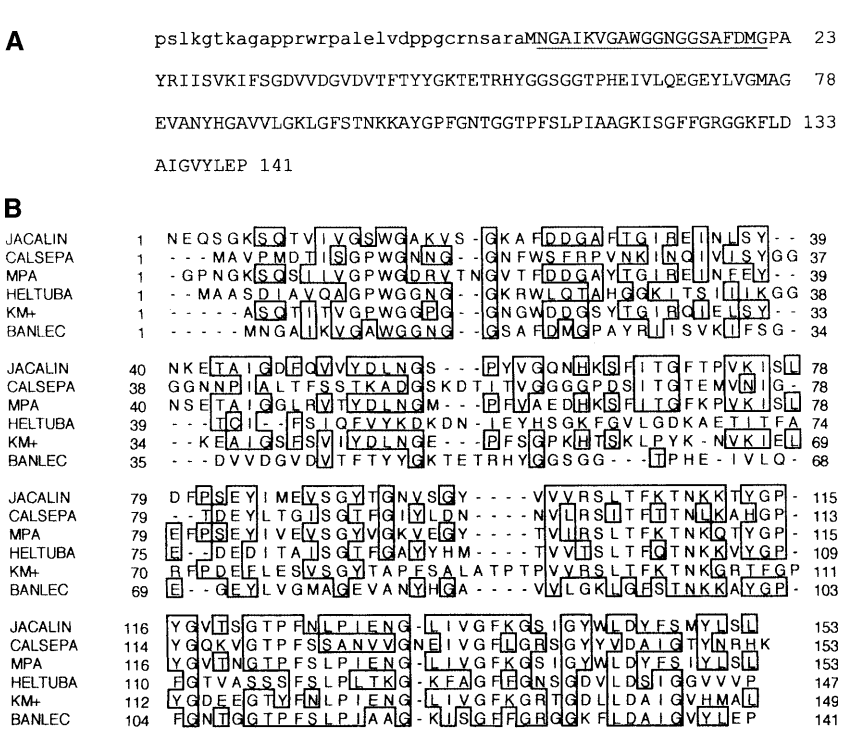

Fig. 3. A Deduced amino acid sequence of cDNA clone pBAN 3-32 encoding BanLec. Since the methionine at position 33 is probably the first amino acid, the residues preceding this methionine are shown in *lower case*. The sequence corresponding to the N-terminal sequence of the protein is *underlined*. **B** Multiple alignment of the amino acid sequences of BanLec, jacalin and other jacalin-related sequences. *JACALIN*, from *Artocarpus integrifolia* (Genbank accession number L03797); *CALSEPA*, from *Calystegia sepium* (Genbank accession number U56820); *MPA*, from *Maclura pomifera*; *HELTUBA,* from *Helianthus tuberosus* (Genbank accession number AF064029); *KM+*, from *Artocarpus integrifolia* (KM+); *BANLEC*, from *Musa acuminata* (this work). Identical residues are *boxed*. *Dashes* correspond to gaps introduced to maximize the homology

comparison, the response to concanavalin A (ConA) was similar in terms of kinetics and magnitude. The magnitude of the response of BALB/c spleen cells was the same when BanLec or ConA were used as stimulators, but the peak of the thymidine uptake was 24 h earlier with ConA. Preliminary flow-cytometry analysis of splenic cells showed that BanLec activated the T-cell population in C57BL/6 and BALB/c mice (data not shown), demonstrating that this lectin is a potent mitogen for murine T-cells.

Molecular cloning of BanLec. A cDNA encoding a putative protein with sequence similarity to jacalin was identified previously in a study of differential gene expression during banana fruit ripening (Clendennen and May 1997). The sequence of this clone pBAN 3-32 (GenBank accession No. AF001527) was completed and analyzed. Sequence analysis of pBAN 3-32 revealed that this cDNA contains an open reading frame of 519 bp encoding a 173-amino-acid precursor with one putative initiation codon at position 33 of the deduced amino acid sequence (Fig. 3A). Translation starting with this methionine residue results in a protein of 141 amino acids with a calculated molecular mass of 14 563 Da and an isoelectric point of 7.68. A comparison of the deduced amino acid sequence of pBAN 3-32 and the N-terminal sequence of BanLec demonstrates a perfect match between residues N34-G53 of the cDNA and the N-terminus of the mature lectin.

No signal peptide could be traced in the deduced amino acid sequence (von Heijne 1986), indicating that BanLec is not synthesized on the endoplasmic reticulum. The apparent absence of a signal peptide is not unique for BanLec but is typical for all members of the subfamily of the mannose-specific jacalin-related lectins. Evidence for the absence of a signal peptide in these proteins has not only been obtained from sequence analysis of cDNAs but also from sequence data of genomic clones. For example, a genomic clone of rice encoding a putative mannose-binding jacalin-related lectin clearly lacks a signal peptide sequence (Garcia et al. 1998). Additional evidence for the absence of a signal peptide from BanLec was obtained from experiments with transgenic tobacco and *Arabidopsis* plants expressing a BanLec construct starting with the N-terminal methionine residue and ending with a BanLec stop codon of the cDNA clone (i.e. a construct from which the 5′ and 3′ untranslated regions of the RNA were removed). Both tobacco and *Arabidopsis* expressed recombinant BanLec, which had the same N-terminal sequence as the lectin isolated from bananas and exhibited identical biological activities (data not shown).

Southern blot analysis. Southern blot analysis showed that a BanLec gene fragment hybridized to several restriction fragments from Grand Nain genomic DNA (Fig. 4) as well as from several other banana cultivars (data not shown). This suggests that the BanLec gene is a member of a multi-gene family as has been found with several other plant lectins (Van Damme et al. 1998).

Induction of lectin with methyl jasmonate. Young banana plants were treated with methyl jasmonate for 3 d and tissue samples of leaves and roots analyzed for the accumulation of lectin. Northern blot analysis of total RNA isolated from leaves and roots of control plants and methyl-jasmonate-treated plants revealed a clear increase in lectin mRNA transcripts in leaves after treatment (Fig. 5). The size of the lectin mRNA was estimated to be approximately 800 nucleotides, which is in good agreement with the size of the cDNA and confirms earlier results by Clendennen and May (1997).

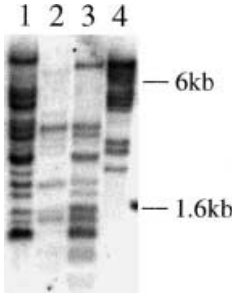

Fig. 4. Southern blot of Grand Nain genomic DNA digested with *Hin*dIII (*lane 1*), *Eco*RI and *Pst*I (*lane 2*), *Eco*RI and *Hin*dIII (*lane 3*) and *Eco*RI (*lane 4*). The positions of several DNA size standards are indicated on the *right*. The blot was probed was a radioactively labeled *Xba*I-*Xho*I fragment of approx. 700 bp from the BanLec gene from the original pBAN 3-32 clone (Clendennen and May 1997)

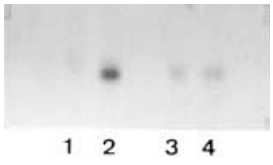

Fig. 5. Northern blot analysis of total RNA isolated from leaves and roots of young banana plants either untreated (*lanes 1*, *3*) or treated (*lanes 2*, *4*) with methyl jasmonate for 3 d. Approximately 40 μg of total RNA was analyzed in each lane. *Lanes 1* and *2* show RNA isolated from leaves. *Lanes 3* and *4* show RNA isolated from roots. The blot was hybridized using the random-primer-labeled pBAN 3-32

The results of the northern blot analysis were confirmed by agglutination tests. Extracts from leaves of untreated plants did not exhibit a detectable agglutination activity (detection limit: 1 μg lectin per g leaf tissue) whereas extracts from methyl-jasmonate-treated plants yielded a clearly visible agglutination activity (corresponding to 2.5 μg lectin per g leaf tissue).

Sequence similarity between BanLec and other lectins. The banana lectin clearly shows sequence similarity with previously isolated jacalin-related lectins from *Calystegia sepium* (Calsepa; Van Damme et al. 1996), *Helianthus tuberosus* (Heltuba; Van Damme et al. 1999), *Artocarpus integrifolia* (jacalin and KM+; Yang and Czapla 1993; Rosa et al. 1999) and *Maclura pomifera* (MPA; Young et al. 1989, 1991). Sequence comparisons indicate that some amino acids are conserved among the different lectin sequences, especially in the C-terminal region of the sequence. It should be noted that in the case of the jacalin sequence the signal peptide and the propeptide of the lectin precursor were removed as well as the linker sequence between the α and β chains.

A phylogenetic tree was built from the available amino acid sequences of isolated jacalin-related lectins. As shown in Fig. 6, clustering of lectins is in good agreement with the gross taxonomical classification since lectins from the Moraceae (jacalin, MPA, KM+) are separated from Convolvulaceae (Calsepa) and Compositae (Heltuba). The banana lectin, BanLec, which belongs to the monocots, is rather distant from the dicot groups.

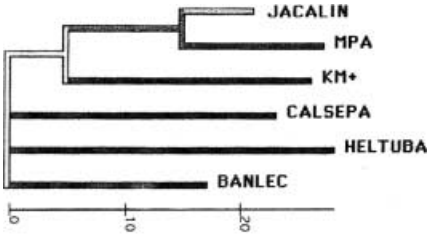

Fig. 6. Phylogenetic tree built from the amino acid sequences of jacalin and jacalin-related sequences. Jacalin from *Artocarpus integrifolia* (*JACALIN*), KM+ from *Artocarpus integrifolia* (*KM+*), Osage orange lectin from *Maclura pomifera* (*MPA*), *Helianthus tuberosus* lectin (*HELTUBA*), *Calystegia sepium* lectin (*CALSEPA*) and banana lectin from *Musa acuminata* (*BANLEC*). Branches of the tree are *shaded* according to the amount of amino acid changes and the *scale* indicates the number of amino acid changes

Molecular modelling. Despite the moderate degree of sequence identity (35%) and homology (57%) between BanLec and Heltuba, both proteins exhibit very similar HCA plots (Fig. 7). Besides an insertion and a deletion of a few residues located at the N-terminal part of the sequence, the 12 strands of anti-parallel β-sheet occurring along the amino acid sequence of Heltuba can easily be delineated in the sequence of BanLec. The three-dimensional model of the BanLec monomer built from the X-ray coordinates of Heltuba, exhibits the β-prism fold (Fig. 8) previously reported for jacalin (Sankaranarayanan et al. 1996) and the *Maclura pomifera* agglutinin MPA (Lee et al. 1998). However, since the BanLec protomer is, unlike the jacalin protomer, not processed into α and β chains, all the strands of β-sheet forming the β-prism of the BanLec monomer are located on a single polypeptide chain. Accordingly, the first strand of β-sheet is covalently linked by a flexible glycine-rich loop to the rest of the polypeptide chain. A similar situation has been reported for KM+, another mannose-binding lectin isolated from jackfruit (*Artocarpus integrifolia*) seeds (Rosa et al. 1999). As an assessment for the validity of the three-dimensional model of banana lectin, all the modelled loops were shown to fall into the generously allowed regions of the Ramachandran plot, except for residue Val^{36} which belongs to the extra-sequence loop located at the N-terminus of the polypeptide chain.

Some of the amino acid residues of jacalin involved in the binding of D-galactose (Sankaranarayanan et al. 1996) are replaced by other residues in the BanLec polypeptide (i.e. Gly^{1}, Tyr^{122}, Trp^{123} and Asp^{125} of the α-chain of jacalin are replaced by Gly^{15}, Lys^{130}, Phe^{131} and Asp^{133} in BanLec; Fig. 9). Despite these changes, the hydrogen bonds to O5 and O6 of the pyranose ring of the monosaccharides are not affected because Asp^{125} is unchanged (Asp^{133} in BanLec) and the H-bonds with Tyr^{122} and Trp^{123} of jacalin (Lys^{130} and Phe^{131} in BanLec) exclusively involve their backbone C=O and =N—H groups. The main change concerns the binding of Gly^{1} of the jacalin α-chain (this residue corresponds to Gly^{21} of the jacalin sequence shown in Fig. 4) to O3 of the pyranose ring, which involves the free amino group generated by the post-

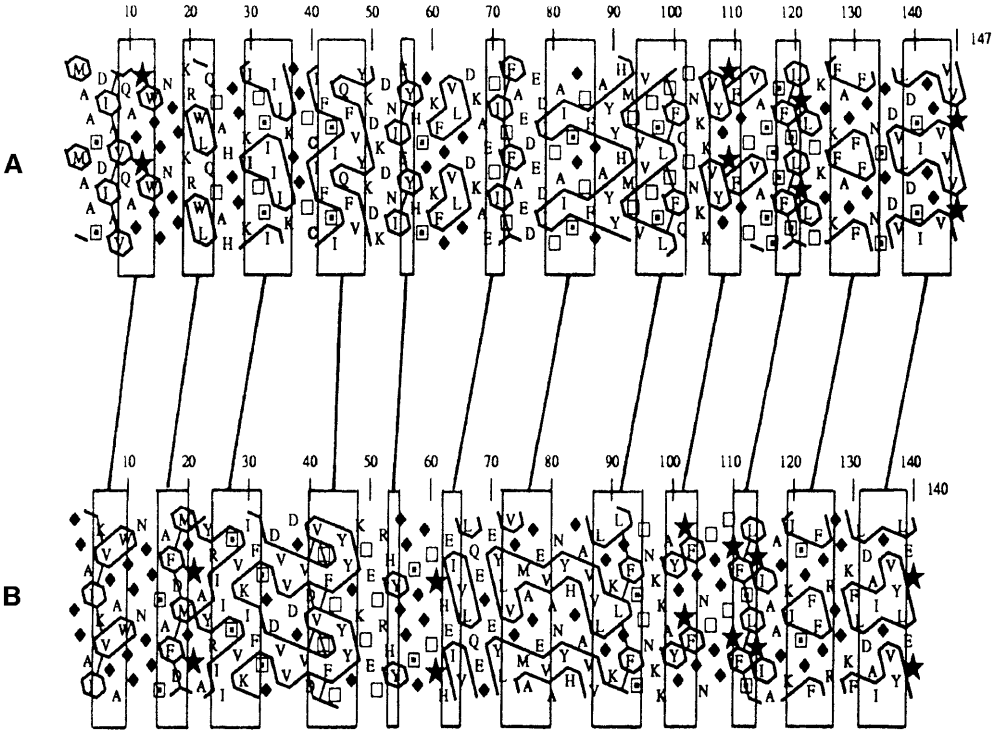

Fig. 7A,B. Comparison of the HCA plots of Heltuba (**A**) and BanLec (**B**). The strands of β-sheet (*boxes*) delineated on the HCA plot of Heltuba were reported on the HCA plot of banana lectin. These delineations were used to recognize the structurally conserved regions of both lectins

translational cleavage of the jacalin precursor into the α and β chains. Since the banana lectin polypeptide is not processed, the =N—H group of Gly^{15} of BanLec (which replaces Gly^{1} of the jacalin α-chain) can not interact with O3. However, the =N—H group (of Gly^{15} of BanLec) remains close enough to the equatorial O3 of mannose to create an hydrogen bond depending on the local folding of the polypeptide chain. In jacalin, Gly^{1} also interacts with the axial O4 of galactose. However, due to the equatorial position of this oxygen in D-glucose or D-mannose, its interaction with Gly^{15} should be different, even though model building performed with D-glucose instead of D-galactose indicates that Asp^{125} of jacalin, another ligand of O4, still interacts with O4 at the equatorial position (Sankaranarayanan et al. 1996). It appears, therefore, that the differences in specificity between BanLec and jacalin can be explained in terms of amino acid substitutions.

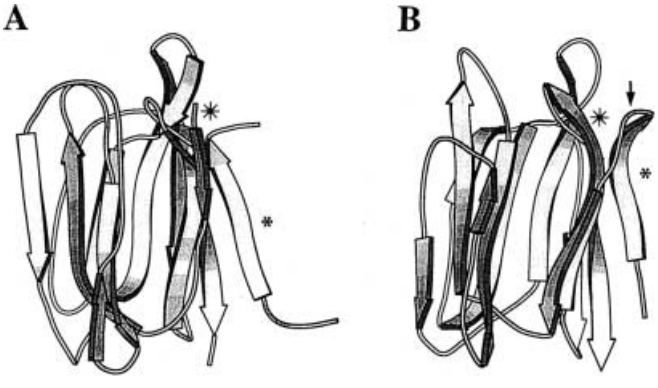

Fig. 8A,B. Side views of the three-dimensional model of the BanLec monomer (**B**) built from the X-ray coordinates of Heltuba, compared to the three-dimensional structure of the jacalin monomer (**A**). The twelve strands of anti-parallel β-sheet (indicated by *arrows*) forming the β-prism fold of the monomer are arranged in three bundles of four strands each. The first strand of β-sheet forming the α-chain of jacalin (which is located on the single polypeptide chain of BanLec) is indicated by an asterisk (*). The arrow (↓) shows the glycine-rich loop which is not proteolytically cleaved in BanLec. Stars (✳) show the location of the carbohydrate-binding sites. Cartoons were rendered with Molscript (Kraulis 1991)

Discussion

A simple straightforward procedure has been developed to isolate the lectins from the pulp of ripe bananas and

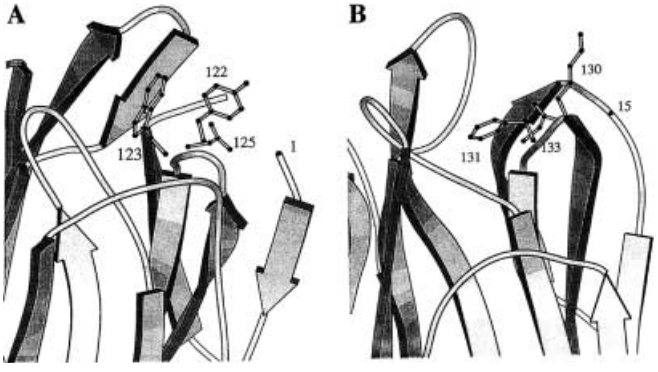

Fig. 9. Amino acid residues forming the carbohydrate-binding sites of jacalin (**A**) and the corresponding residues of BanLec (**B**). Due to the cleavage occurring in jacalin, Gly^{1} of the α-chain possesses a free $-NH_2$ group whereas the corresponding residue Gly^{15} of BanLec, which is not cleaved, exhibits no free $-NH_2$ group. Cartoons were rendered with Molscript (Kraulis 1991)

plantains. The combined results of the purification of the protein and sequencing of the corresponding cDNA clearly demonstrate that BanLec is a member of the family of jacalin-related lectins. The two lectins BanLec and PlanLec are the first documented examples of jacalin-related lectins, which are highly expressed in fruits but are, at least under normal conditions (i.e. without a specific treatment of the plants), absent from or present in very low concentrations in other tissues. According to an earlier study of the differential gene expression in ripening banana fruit the lectin mRNA is down-regulated during fruit ripening. Northern blot analysis further indicated that the mRNA encoding BanLec is expressed in the pulp and roots but not in the peel, leaf and corm (Clendennen and May 1997). Agglutination assays with extracts from different tissues confirmed the results of these northern blot analyses. No activity could be detected in extracts from peels, leaves and corms but extracts from roots and pulp showed a weak but clearly detectable agglutination activity corresponding to a lectin content of about 0.5 and 3 μg lectin per g fresh weight, respectively. Interestingly, the banana lectin was induced in leaves by methyl jasmonate which suggests that it might be expressed as a response to abiotic or biotic stress factors. This finding is important because it demonstrates for the first time that the plant hormone methyl jasmonate effectively induces the accumulation of a biologically active plant lectin.

Southern blot analysis demonstrated that BanLec is controlled by a multigene family. Apparently the BanLec gene family is far more complex than the small gene family encoding the mannose-specific jacalin-related lectin from, for example, *Calystegia sepium* and *Helianthus tuberosus*. In this respect, BanLec resembles the unrelated monocot mannose-binding lectins from, for example, species of the Amaryllidaceae and Alliaceae that are also encoded by highly complex gene families (Van Damme et al. 1998).

The banana lectin is a potent mitogen for murine T-cells although the level of proliferation differs between strains of mice. The banana lectin has also been shown to stimulate human T-cell proliferation even at a dose as low as 0.3 μg/ml (Koshte et al. 1990). Considering the fact that banana pulp contains at least 10 μg highly stable and protease-resistant BanLec per gram fresh weight, the threshold concentration for mitogenic activity can certainly be reached in the small intestine after ingestion of bananas. Consequently, eating bananas can in principle lead to an immune response. The latter assumption is supported by the earlier observation that IgG4 antibodies against banana lectin are found with an unexpectedly high frequency in human serum (Koshte et al. 1992). Though the effects of orally ingested banana lectin on humans are not necessarily negative, it is important to realize that this major fruit crop contains relatively high concentrations of a mannose-specific lectin. It is interesting to refer in this respect to the controversy that has arisen about the presumed adverse effects of transgenic potatoes expressing the mannose-specific snowdrop (*Galanthus nivalis*) agglutinin on rats (Ewen and Pusztai 1999).

The presence of relatively large quantities of mannose-specific jacalin-related lectins in ripe banana and plantain fruits raises the question of the physiological role of these fruit-associated biologically active proteins. Some members of the subfamily of the galactose-binding jacalin-related lectins (like jacalin and the *Maclura pomifera* lectins) possess insecticidal properties and hence may be involved in plant defense against insects. Hitherto, no such activity has been reported for any of the mannose-binding jacalin-related lectins. It is questionable, therefore, whether BanLec and PlanLec are anti-insect proteins. Possibly, the role of the banana lectin is based on its capability to stimulate animal lymphocytes. One can imagine, for example, that BanLec exhibits a differential effect according to the glycans present on the surface of the immunocompetent cells in the gut of different animal. If so, the lectin may be selectively toxic for or deter those fructifagous species that do not contribute to the dissemination of the seeds. As a result, the fruits are only eaten by animals that are likely to disseminate the seeds at the right time and at the right place, and hence contribute to the survival of the species in its natural habitat.At present, little is known about the role of the mannose-specific jacalin-related lectins. To pursue functional studies it is a prerequisite to get an overview of the occurrence of these lectins within the plant kingdom, their tissue-specific expression and their regulation by external factors. In addition, it will be of crucial importance to identify the natural glycan-receptors of these lectins. Since hitherto only three other mannose-specific jacalin-related lectins have been characterized, the identification and characterization of BanLec is highly relevant because further evidence is provided that these lectins exhibit differences in specificity and biological activities in spite of their high sequence similarity. Recently, we have shown that the gene product of a salt-stress-inducible gene from rice (SalT) corresponds to a mannose-specific jacalin-related lectin (Zhang et al. 2000). This finding clearly indicates that at least some members of this lectin family are stress-related proteins. The possible role of other mannose-specific jacalin-related lectins in stress responses is currently being studied.

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References

Bemer V, Truffa-Bachi P (1996) T cell activation by concanavalin A in the presence of cyclosporin A: immunosuppressor withdrawal induces NFATp translocation and interleukin-2 gene transcription. Eur J Immunol 26: 1481–1488

Bourne Y, Zamboni V, Barre A, Peumans WJ, Van Damme EJM, Rougé P (1999) Crystal structure of *Helianthus tuberosus* lectin,

a widespread scaffold for mannose-binding lectins. Structure Fold Des 7: 1473–1482

Clendennen SK, May GD (1997) Differential gene expression in ripening banana fruit. Plant Physiol 115: 463–469

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugar and related substances. Anal Chem 28: 350–356

Eggermont K, Goderis IJ, Broekaert WF (1996) High-throughput RNA extraction from plant samples based on homogenisation by reciprocal shaking in the presence of a mixture of sand and glass beads. Plant Mol Biol Rep 14: 273–279

Ewen SWB, Pusztai A (1999) Effect of diets containing genetically modified potatoes expressing *Galanthus nivalis* lectin on rat small intestine. Lancet 354: 1353–1354

Gaboriaud C, Bissery V, Benchetrit T, Mornon JP (1987) Hydrophobic cluster analysis: an efficient new way to compare and analyse amino acid sequences. FEBS Lett 224: 149–155

Garcia AB, Engler JA, Claes B, Villarroel R, Van Montagu M, Gerats T, Caplan A (1998) The expression of the salt-responsive gene *salT* from rice is regulated by hormonal and developmental cues. Planta 207: 172–180

Geshi N, Brandt A (1998) Two jasmonate-inducible myrosinase-binding proteins from *Brassica napus* L. seedlings with homology to jacalin. Planta 204: 295–304

Koshte VL, Van Dijk W, Van Der Stelt ME, Aalberse RC (1990) Isolation and characterization of BanLec-I, a mannoside-binding lectin from *Musa paradisiac* (banana). Biochem J 272: 721–726

Koshte VL, Aalbers M, Calkhoven PG, Aalberse RC (1992) The potent IgG4-inducing antigen in banana is a mannose-binding lectin, BanLec-I. Int Arch Allergy Immunol 97: 17–24

Kraulis PJ (1991) Molscript: a program to produce both detailed and schematic plots of protein structures. J Appl Cryst 24: 946–950

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227: 680–685

Lee X, Thompson A, Zhang Z, Ton-that H, Biesterfeldt J, Ogata C, Xu L, Johnston AZ, Young NM (1998) Structure of the complex of *Maclura pomifera* agglutinin and the T-antigen disaccharide, Galβ1, 3GalNAc. J Biol Chem 273: 6312–6318

Lemesle-Varloot L, Henrissat B, Gaboriaud C, Bissery V, Morgat A, Mornon JP (1990) Hydrophobic cluster analysis: procedure to derive structural and functional information from 2-D-representation of protein sequences. Biochimie 72: 555–574

Maddison WP, Maddison DR (1992) MacClade: analysis of phylogeny and character evolution, Version 3.0. Sinauer Associates, Sunderland, Massachusetts (USA)

May GD, Afza R, Mason HS, Wiecko A, Novak FJ, Arntzen CJ (1995) Generation of transgenic banana (*Musa acuminata*) plants via *Agrobacterium*-mediated transformation. Biotechnology 13: 486–492

Peumans WJ, Winter HC, Bemer V, Van Leuven F, Goldstein IJ, Truffa-Bachi P, Van Damme EJM (1997) Isolation of a novel plant lectin with an unusual specificity from *Calystegia sepium*. Glycoconjugate J 14: 259–265

Rosa JC, De Oliveira PSL, Garratt R, Beltramini L, Resing K, Roque-Barreira M-C, Greene LJ (1999) KM+, a mannose-binding lectin from *Artocarpus integrifolia*: amino acid sequence, predicted tertiary structure, carbohydrate recognition, and analysis of the β-prism fold. Protein Sci 8: 13–24

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467

Sankaranarayanan R, Sekar K, Banerjee R, Sharma V, Surolia A, Vijayan M (1996) A novel mode of carbohydrate recognition in jacalin, a Moraceae plant lectin with a β-prism fold. Nature Struct Biol 3: 596–603

Sastry MVK, Banerjee P, Patanjali SR, Swamy MJ, Swarnalatha GV, Surolia A (1986) Analysis of the saccharide binding to *Artocarpus integrifolia* lectin reveals specific recognition of T-antigen (β-D-Gal(1,3)D-GalNAc). J Biol Chem 261: 11726–11733

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680

Van Damme EJM, Barre A, Verhaert P, Rougé P, Peumans WJ (1996) Molecular cloning of the mitogenic mannose/maltose-specific rhizome lectin from *Calystegia sepium*. FEBS Lett 397: 352–356

Van Damme EJM, Peumans WJ, Barre A, Rougé P (1998) Plant lectins: a composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. Crit Rev Plant Sci 17: 575–692

Van Damme EJM, Barre A, Mazard A-M, Verhaert P, Horman A, Debray H, Rougé P, Peumans WJ (1999) Characterization and molecular cloning of the lectin from *Helianthus tuberosus*. Eur J Biochem 199: 135–142

von Heijne G (1986) A method for predicting signal sequence cleavage sites. Nucleic Acids Res 11: 4683–4690

Yang H, Czapla TH (1993) Isolation and characterization of cDNA clones encoding jacalin isolectins. J Biol Chem 268: 5905–5910

Young MN, Johnston RAZ, Szabo AG, Watson DC (1989) Homology of the D-galactose-specific lectins from *Artocarpus integrifolia* and *Maclura pomifera* and the role of an unusual small polypeptide unit. Biochim Biophys Acta 270: 596–603

Young NM, Johnston RAZ, Watson DC (1991) The amino acid sequences of jacalin and the *Maclura pomifera* agglutinin. FEBS Lett 282: 382–384

Zhang W, Peumans WJ, Barre A, Houles-Astoul C, Rovira P, Rougé P, Proost P, Truffa-Bachi P, Jalali HA, Van Damme EJM (2000) Isolation and characterization of a jacalin-related mannose-binding lectin from salt-stressed rice (*Oryza sativa*) plants. Planta, in press